IDENTIFICATION OF AN ENDOGENOUS Con A-BINDING POLYPEPTIDE AS THE HEAVY SUBUNIT OF α-MANNOSIDASE

D. J. BOWLES, J. ANDRALOJC and S. MARCUS
Department of Biochemistry, University of Leeds, Leeds LS2 9JT, England

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1. Introduction

We showed in [1] that the specificity of endogenous lectins for glycoproteins in homologous plant species was extremely high. In jackbean cotyledons, a major polypeptide recognised by concanavalin A (Con A) had an M_r app. ≈ 68 000 and could be purified from an aqueous-buffered extract by affinity chromatography on immobilised Con A. When we assayed glycosidase activities in extracts of cotyledons and embryonic axes, we found that the only glycosidase with significantly different activity in the 2 tissues was α-mannosidase [2,3]. α-Mannosidase activity was extremely high in the cotyledon extract and virtually absent from the axis extract. This tissue distribution paralleled that of the endogenous lectin Con A [1,3] and suggested the possibility that the 2 seed components were in some way related. Here, we suggest that the major Con A binding polypeptide in jackbean cotyledons is the heavy subunit of α-mannosidase.

2. Methods

Extracts were prepared from tissues of seeds imbibed for 6 h in H_2O containing or not, 5 mM $CuCl_2$ by sonication in 10 mM Tris–HCl (pH 7.0), 1 mM $CaCl_2$, 1 mM $MgCl_2$ containing or not 5 mM $CuCl_2$. Soluble extracts were prepared by centrifugation at 10 000 × *g* for 4 min (Eppendorf microfuge). α-Mannosidase and Con A (type 4) were purchased from Sigma. Protein was determined by the Coomassie dye procedure [4]. Samples for SDS–polyacrylamide gel electrophoresis (SDS–PAGE), were precipitated by addition of 5 vol. excess of −20°C acetone and incubation in ice for 15 min. The precipitates were solubilised in sample buffer as in [1] and electrophoresed in the presence of SDS and dithiothreitol on a 10% (w/v) polyacrylamide gel. Con A was radiolabelled in the presence of 0.2 M methyl-α-D-mannoside, with ^{125}I (Amersham spec. act. 16.9 mCi/μg) using Enzymobeads (Biorad) and separated from free ^{125}I by passage through Biogel P_6. Carbohydrate binding activity of the ^{125}I-labelled lectin, as estimated by sugar-inhibitable haemagglutination, was unimpaired (not shown). After staining the polypeptides separated by SDS–PAGE with Coomassie blue and destaining, the gels were incubated in PBS: phosphate-buffered saline (pH 7.2), 0.5% sodium azide, with 3 changes for 24 h at room temperature. They were then further incubated in PBS, 1 mg/ml bovine serum albumin (BSA) (Sigma) containing or not, 0.2 M methyl-α-D-mannoside with 2 changes for 12 h at 4°C. ^{125}I-labelled Con A was then added to the final solutions to give 10^6 cpm/ml and the gels were incubated with shaking at 4°C for 4 h after which the ^{125}I-containing solution was removed and the gels were further washed in PBS, 0.5% sodium azide, 1 mg BSA/ml, containing or not 0.2 M methyl-α-D-mannoside. When the radioactivity in the wash solutions was at background level, the gels were dried and exposed to X-ray film within intensifier screens at −70°C and the films developed after 1 week's exposure.

3. Results and discussion

Fig.1 shows the results of jackbean extracts analysed by SDS–PAGE and compares Coomassie blue staining for protein with an autoradiograph of the same gel after an overlay with ^{125}I-Con A in the absence of methyl-α-D-mannoside. Since we were

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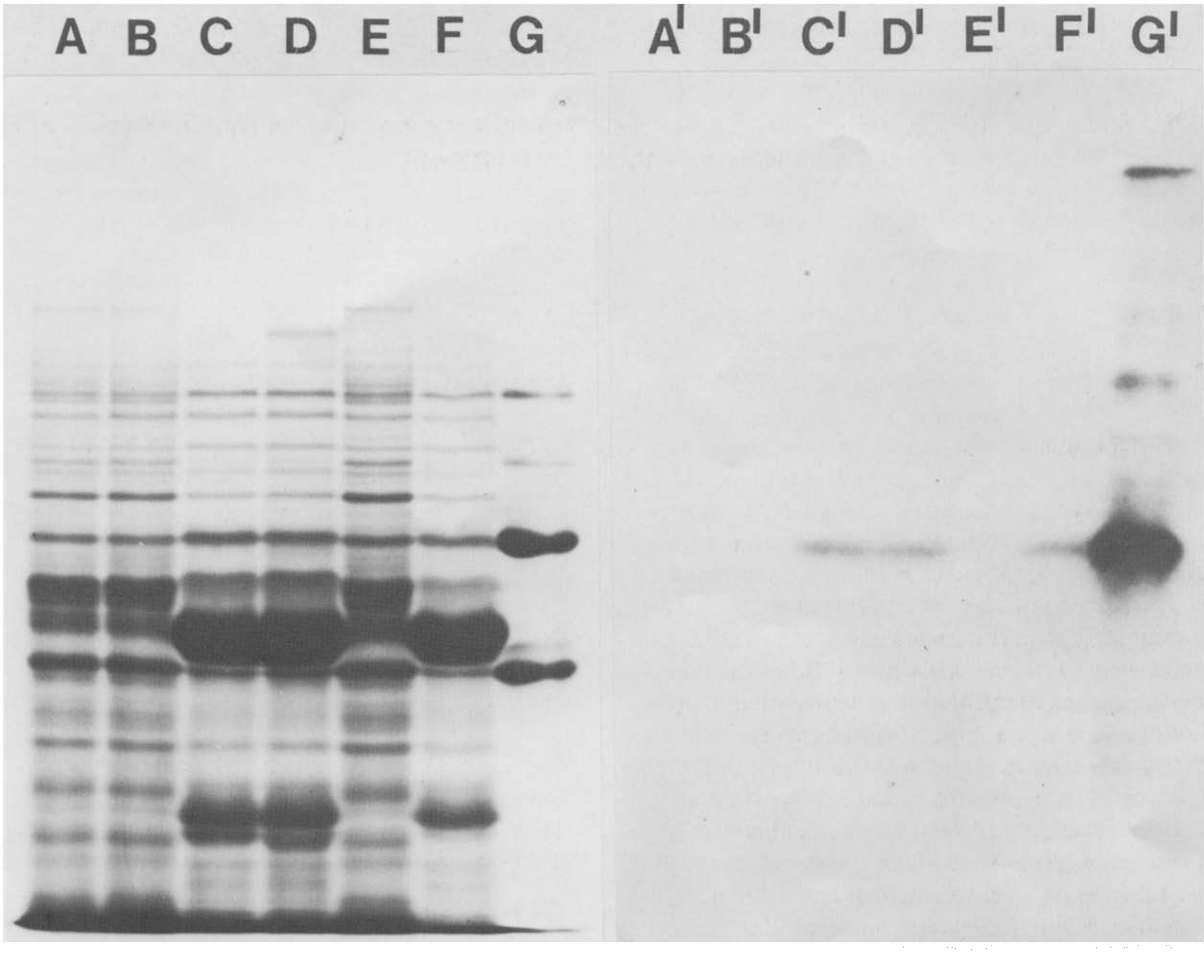

Fig.1. SDS–PAGE of jackbean seed extracts and purified α-mannosidase. Comparison of Coomassie blue staining for protein and autoradiograph of the gel after overlay with ^{125}I-Con A. Extracts were prepared as in section 2 and electrophoresed in the presence of SDS and dithiothreitol on a 10% (w/v) polyacrylamide gel. (A–G) Polypeptides stained with Coomassie blue; (A′–G′) autoradiographs of the same gels after overlay with ^{125}I-Con A as in section 2; (A,A′) total extract of embryonic axis; (B,B′) as (A) but prepared in presence of 5 mM Cu^{2+}; (C,C′) total extract of cotyledon; (D,D′) as (C) but prepared in presence of 5 mM Cu^{2+}; (E,E′) soluble extract of embryonic axis; (F,F′) soluble extract of cotyledon; (G,G′) α-mannosidase (Sigma); (A–F) 100 μg protein loaded/channel, (G) 25 μg protein loaded.

interested in analysing Con A-specific mannosyl residues attached to polypeptides, it was important to prevent any hydrolysis by exoglycosidases and endopeptidases also present in the homogenate. Consequently, extracts were prepared in the presence or absence of 5 mM Cu^{2+}. This [Cu^{2+}] inhibits the activity of purified α-mannosidase [5], activity of the enzyme in total extracts of jackbean cotyledons [3] and activity of the endogenous proteases of jackbean seeds (K. Dalkin, D. J. B., in preparation). From the Coomassie blue staining of the 10% gel, no major differences are observed for the pattern of polypeptides in total extracts prepared in the presence or absence of 5 mM Cu^{2+}. Similarly, the overall pattern is very similar for total and soluble extracts, confirming that the sonication procedure solubilises the majority of jackbean seed polypeptides as suggested [1]. α-D-Mannosidase is composed of 2 types of subunits [6] which, in our gel system, have M_r values of ~68 000 and ~45 000. The native enzyme is a tetramer [6,7] and therefore most probably contains 2 subunits of 68 000 M_r and 2 subunits of 45 000 M_r [6]. The Sigma preparation of the enzyme can be further purified using DEAE-cellulose [8] leading to removal

of some of the additional contaminant popypeptides visualised after SDS–PAGE (not shown).

When the gel was analysed by overlay with ^{125}I-Con A under the conditions in section 2, the subsequent autoradiograph revealed that the heavy subunit of α-mannosidase bound ^{125}I-Con A. In addition, a single polypeptide in the cotyledon extracts also bound ^{125}I-Con A and electrophoresed with an identical apparent M_r to the heavy subunit of α-mannosidase. The number of Con A-binding polypeptides did not increase in the presence of 5 mM Cu^{2+}, suggesting that the high specificity exhibited by the endogenous lectin was most probably not caused by hydrolysis of mannosyl residues during extract preparation. Under the conditions used, no polypeptides in the embryonic axis extract were radioactively labelled by the ^{125}I-Con A. When an identical gel was preincubated, incubated in ^{125}I-Con A and washed in the presence of 0.2 M methyl-α-D-mannoside, the subsequent autoradiograph revealed an absence of radioactively labelled polypeptides, indicating that the interaction of ^{125}I-Con A with the jackbean components in the gel was most probably via the mannosyl-binding activity of the lectin (not shown). These characteristics clearly distinguish the Con A-binding polypeptide described here and in [1] from that in [9] where app. M_r was 35 000, and Con A binding to the polypeptide was inhibited by NaCl at >5 mM.

These results indicate that the close relationship between Con A and α-mannosidase implied by their parallel tissue distribution in the seed [3] is confirmed by the finding that a subunit of α-mannosidase can interact with Con A. Both subunits of jackbean α-mannosidase are glycosylated on the basis of PAS-staining [6] and α-mannosidase from *Phaselus vulgaris* contains mannose [10]. Elsewhere, we present GLC evidence that jackbean α-mannosidase is mannosylated and results which indicate the affinity of Con A for the heavy subunit is much greater than that of the lectin for the native enzyme [11]. Why a mannose-binding lectin should interact with a mannose-hydrolysing enzyme and, why a mannose-hydrolysing enzyme should be highly-mannosylated are under investigation.

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